MOLECULAR AND CELLULAR BIOLOGY, Dec. 2003, p. 8786–8794 Vol. 23, No. 23
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.23.8786–8794.2003

Antioxidants Enhance Mammalian Proteasome Expression through the Keap1-Nrf2 Signaling Pathway

Mi-Kyoung Kwak,[1] Nobunao Wakabayashi,[1,2] Jennifer L. Greenlaw,[1] Masayuki Yamamoto,[2] and Thomas W. Kensler[1]*

Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland 21205,[1] and Institute of Basic Medical Sciences and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tennoudai, Tsukuba 305-8577, Japan[2]

Received 20 June 2003/Returned for modification 6 August 2003/Accepted 26 August 2003

Proteasomes degrade damaged proteins formed during oxidative stress, thereby promoting cell survival. Neurodegenerative and other age-related disorders are associated with reduced proteasome activity. We show herein that expression of most subunits of 20S and 19S proteasomes, which collectively assemble the 26S proteasome, was enhanced up to threefold in livers of mice following treatment with dithiolethiones, which act as indirect antioxidants. Subunit protein levels and proteasome activity were coordinately increased. No induction was seen in mice where the transcription factor Nrf2 was disrupted. Promoter activity of the PSMB5 subunit of the 20S proteasome increased with either Nrf2 overexpression or treatment with antioxidants in mouse embryonic fibroblasts. Tandem antioxidant response elements in the proximal promoter of *PSMB5* that controlled these responses were identified. We propose that induction of the 26S proteasome through the Nrf2 pathway represents an important indirect action of these antioxidants that can contribute to their protective effects against chronic diseases.

Accumulation of abnormal proteins in cells impedes cellular function and can lead cells to apoptosis (6, 39). 26S proteasomes are responsible for the degradation of damaged or misfolded proteins and control levels of key regulatory molecules (6, 15). The proteasome is a large multisubunit complex that contains a proteolytic active 20S core complex consisting of a cylindrical stack of four rings (15). Two inner rings formed with seven β-subunits have proteolytic activity while two outer rings of α-subunits maintain structure. Access to the inner facet of the cylinder is controlled through gating by a 19S regulatory subunit attached to one or both ends. The 19S proteasome participates in the recognition and processing of substrates before their translocation and degradation by the catalytic core (Fig. 1A). The 20S proteasome can directly degrade oxidized proteins, while ubiquitination marks many proteins for recognition and turnover by the entire 26S complex (6). Inhibition of proteasome function induces apoptosis in cancer cells and represents a promising molecular target for oncolytic drugs (1). However, a decreased capacity for protein degradation is related to several neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, in which accumulation of abnormal polypeptides within cells leads to death of neurons, as well as diabetes and atherosclerosis. An altered ubiquitin-proteasome system and reduced proteasome activity are associated with some of these diseases (8, 17, 22, 34). Antioxidants can neutralize oxidative challenges directly by intercepting free radicals (e.g., vitamins C and E) or indirectly by modulating the expression of genes that detoxify these reactive intermediates or eliminate their damage products (9, 10). Sulforaphane and 3*H*-1,2-dithiole-3-thione (D3T), both of which are isolated from cruciferous vegetables (23, 49), as well as the food antioxidant ethoxyquin, activate transcription of protective genes through the antioxidant response element (ARE). The ARE (5′-TGA[C/T]NNN GC-3′) is a *cis*-acting element governing the regulation of multiple phase 2 genes encoding proteins that protect against oxidative and electrophilic stresses, such as glutathione *S*-transferases, γ-glutamylcysteine ligases, and NADPH quinone oxidoreductase (NQO1) (46). The transcription factors that activate the ARE have been extensively studied; the CNC-bZIP ("cap'n'collar" family of basic leucine zipper) protein Nrf2 is an essential element in the transcription complex of the ARE. Studies using *nrf2*-disrupted mice have clearly demonstrated that Nrf2 is a critical factor in the regulation of many cytoprotective genes (19, 25, 43). Induction of these genes by sulforaphane and D3T is largely attenuated in *nrf2*-disrupted mice (25, 43). Moreover, these antioxidants, which are under development as cancer-preventive agents in humans (24, 38), lose their protective efficacy in the *nrf2* knockout mice (11, 35). Because of their altered transcriptional programming, *nrf2*-disrupted mice are considerably more sensitive to the acute and chronic toxicities of environmental chemicals and hyperoxia (2–4, 35). Nrf2 itself is regulated by Keap1, an actin-binding protein that sequesters Nrf2 in the cytoplasm by specific binding to its amino-terminal regulatory domain (20). Indirect antioxidants cause the dissociation of Nrf2 from Keap1, allowing for nuclear accumulation of Nrf2 and enhanced expression of cytoprotective genes. Additional studies of cell systems and *keap1*-disrupted mice demonstrate that the Keap1-Nrf2 complex is a key sensor regulating expression of genes promoting cell survival (44a).

A microarray-based survey of D3T-inducible genes indicated

* Corresponding author. Mailing address: Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, 615 N. Wolfe St., Baltimore, MD 21205. Phone: (410) 955-4712. Fax: (410) 955-0116. E-mail: tkensler@jhsph.edu.

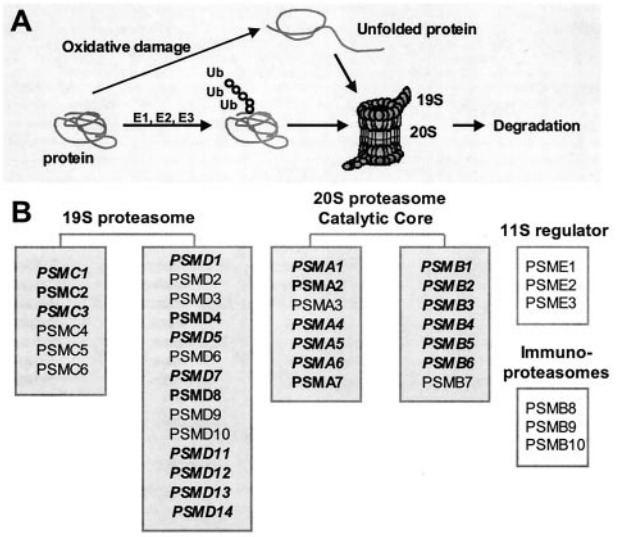


FIG. 1. Microarray summary of proteasome subunits induced in mouse liver by D3T. (A) Proteasomes degrade oxidatively damaged proteins as well as ubiquitin-marked proteins. (B) Subunit components of the 19S and 20S proteasomes. Subunits in boldface italics are genes elevated by D3T in livers of wild-type, but not *nrf2*-disrupted, mice. Subunits in boldface only are genes induced by D3T in both genotypes.

that several hundred genes potentially involved in protection against electrophiles and oxidants were coordinately upregulated (27). Among these genes, multiple proteasome subunits were induced (Fig. 1B). Here we demonstrate that indirect antioxidants, including D3T, increase expression of multiple proteasome subunits through the Keap1-Nrf2-ARE signaling pathway and enhance the activity of the 26S proteasome. Our results indicate that the proteasome genes are prominent downstream targets of the Nrf2 pathway that promotes cell survival.

MATERIALS AND METHODS

Animals and treatment. Wild-type and *nrf2*-disrupted mice were generated from inbred *nrf2* heterozygous mice (25). Mice (10 to 12 weeks old) were fed AIN-76A semipurified diet and treated with D3T (0.5 mmol/kg of body weight) by gavage in a suspension consisting of 1% Cremophor and 25% glycerol. Mice were sacrificed 24 h after treatment, and livers were harvested and snap-frozen. Animal protocols were approved by the Johns Hopkins Animal Care and Use Committee.

Reverse transcription-PCR (RT-PCR) analysis. For the synthesis of cDNAs, 50 ng of total RNA was incubated for 20 min with a solution containing 10 mM Tris (pH 8.4), 5 mM KCl, 5 mM $MgCl_2$, 4 mM deoxynucleoside triphosphates, 0.125 μg of oligo$(dT)_{12-18}$, and 30 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, N.Y.). PCR amplification for each gene was performed with a Fail Safe PCR kit (Epicentre, Madison, Wis.) using a DNA thermal cycler (MJ Research, Watertown, Mass.). Amplification conditions were 26 or 27 cycles of 5 min at 95°C, 30 s at 56°C, and 40 s at 72°C. Primers were synthesized by Integrated DNA Technology (Coralville, Iowa) and were as follows: *PSMA1*, 5′-TGTTTGACAGACCACTTCCT-3′ and 5′-TCTTCAAGACCATCCAGGAA-3′; *PSMA4*, 5′-TGATGCTAACGTTCTGAC-3′ and 5′-TTCAACATTGACACAGCC-3′; *PSMB3*, 5′-TTCAGCGTCCTGGTGGTGAT-3′ and 5′-ACAGAGCCTGTCATTGCTGG-3′; *PSMB5*, 5′-GCTGGCTAACATGGTGTATCAT-3′ and 5′-AAGTCAGCTCATTGTCACTGG-3′; *PSMB6*, 5′-GAGGGCAGGTGTACTCTGTT-3′ and 5′-CAAAACACCTGCCGCTCTA-3′; *PSMB8*, 5′-ATGATGCTGCAGTACCGG-3′ and 5′-CCGTCTTCCTTCATGTG-3′; *PSMC1*, 5′-GTCACAGTGATGAAGGTGGA-3′ and 5′-ACTTTCATTCTGCGTTCCCG-3′; *PSMC3*, 5′-CAAACGCTTCGACAGTGA-3′ and 5′-CTGGGCTCCATTGAAGTC-3′; *PSMD14*, 5′-TATCAACACTCAGCAGAGCT-3′ and 5′-AATCCTTCCATCCAACTCT-3′.

Preparation of tissue homogenates and immunoblot analysis of proteasome subunits. Livers were homogenized with a Dounce homogenizer in buffer containing 50 mM Tris-HCl (pH 7.8), 200 mM KCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol and centrifuged at 9,000 × *g* for 15 min at 4°C. Protein concentration was determined by the bicinchoninic acid assay (Pierce Inc., Rockford, Ill.), and tissue homogenates were loaded on a sodium dodecyl sulfate-polyacrylamide gel and separated by electrophoresis. Gels were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) at 50 V for 3 h, and immunoblotting was carried out with antibodies against PSMA1, PSMB5, and PSMC1 (Research Diagnostics, Inc., Flanders, N.J.). Immunoblotted membranes were developed by using the ECL Western blotting system (Amersham Pharmacia Biotech Inc.) according to the manufacturer's instructions.

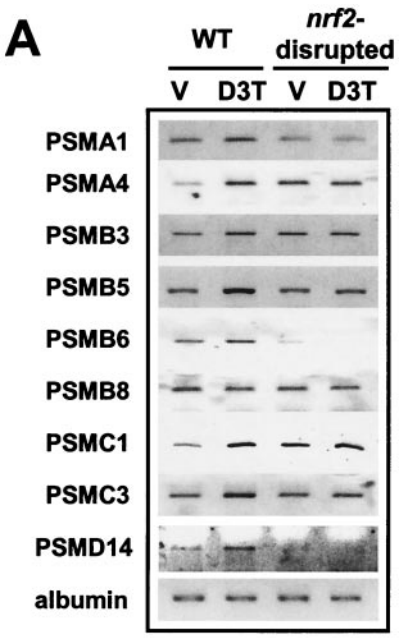
Proteasome activity measurement. Peptidase activity of the proteasome was measured by mixing tissue homogenate with 50 μM fluorogenic peptide Suc-LLVY-AMC (succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin), Z-LLE-AMC (Z-Leu-Leu-Glu-7-amino-4-methylcoumarin), or Z-ARR-AMC (Z-Ala-Arg-Arg-7-amino-4-methylcoumarin) (Calbiochem, La Jolla, Calif.) in a final volume of 100 μl. The reaction buffer consisted of 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol (36). The mixture was incubated at 37°C for 20 min, and then the reaction was stopped by adding an equal volume of 125 mM sodium borate buffer (pH 9.0) containing 7.5% ethanol. Released fluorogenic AMC was measured at 360-nm excitation and 460-nm emission. Fluorescence units were converted to AMC concentration by using standard curves generated from free AMC.

Plasmids. The promoter region of *PSMB5* (from −3414 to −1; NT 039606) was isolated by PCR amplification from hepatic genomic DNA of ICR mice. The isolated PCR product was ligated into the luciferase reporter vector pGLbasic (Promega, Madison, Wis.). Deleted sequences of the *PSMB5* promoter (−1.1kb-luc, −1080 to −1; −0.5kb-luc, −497 to −1; −0.2kb-luc, −211 to −1) were produced by PCR amplification and inserted into the pGL3 basic vector. Two other truncated promoters [−3.4-del (−1.1)-luc, −3414 to −1079; −0.5kb-del (−0.2)-luc, −497 to −210] were amplified by PCR and ligated into the pGL3 promoter vector, which contains the simian virus 40 promoter as a minimal promoter. Mutated *PSMB5* promoters Mut (−341), Mut (−52), and Mut (−341, −52) were generated by PCR using primers containing the mutated −341 ARE (GCCTGGGCAGTGACCAAAC→GCCTGGGTGGCACCAAAC) or −52 ARE (TGACGTCGCGGCGTTGCCA→CAACGTCGCGGCGTTGCTG) (mutated nucleotides are underlined) as described previously (26). The sequence of each promoter was verified.

Cell culture and treatment. Embryonic fibroblast cells from 13.5-day-old embryos of mice with wild-type, *nrf2*-disrupted, and *keap1*-disrupted genotypes were immortalized as described previously (44). Cells were maintained in Iscove's modified Dulbecco's media (Life Technologies) containing 10% heat-inactivated fetal bovine serum and antibiotics.

Transient transfection and measurement of luciferase activity. Cells were transfected at 30 to 40% confluence by Lipofectamine Plus reagent (Life Technologies Inc.). Briefly, cells were seeded in 24-well plates at a density of 2×10^4 cells/well. Cells were grown overnight; the transfection complex containing 0.5 μg of plasmid DNA, 0.05 μg of the pRLtk plasmid (Promega), and transfection reagent were added to each well, and cells were incubated for 16 to 18 h. Cells were recovered in normal media after removal of transfection reagents and were then incubated for another 16 to 18 h with or without drug treatment (D3T or sulforaphane). *Renilla* and firefly luciferase activities in cell lysates were measured with the Dual Luciferase assay kit (Promega) with a luminometer (Turner Designs). For overexpression studies, pcDNA3-murine Nrf2 or -murine MafK was cotransfected with promoter plasmids (26).

Chromatin immunoprecipitation assay. Formaldehyde cross-linking and chromatin fragmentation were carried out as described previously (26). Ten percent of the diluted chromatin solution was reserved as the total input of chromatin. The remaining diluted chromatin solution was incubated with an anti-Nrf2 antibody, an anti GATA-1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.), nonspecific immunoglobulin G, or no antibody for 18 h at 4°C with rotation. After washing and elution, precipitated DNA was resuspended with 30 μl of water, and 1 μl of DNA was used for 30 to 35 cycles of PCR amplification with the following primers: *PSMB5* −341 ARE (5′-TTGAACCAGGATTAGGATAGGTGG-3′ and 5′-CCATCTTTGAGAAGGGCGTAA-CTG-3′) and *PSMB5* −52 ARE (5′-CAGACCGGCGCTGGTATTTAGAGG-3′ and 5′-TAGCCAGCGCCATGTTTAGCAAGG-3′).



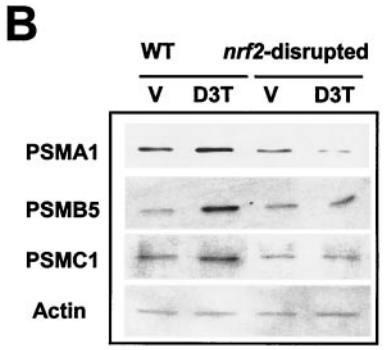


FIG. 2. Induction of 26S transcript and protein levels of subunits by D3T in livers of wild-type mice. (A) mRNA levels for 26S proteasome subunits. Hepatic transcript levels of each subunit in wild-type and *nrf2*-disrupted mice 24 h after treatment with D3T were measured by RT-PCR analysis. Each sample was prepared from three pooled livers. (B) Induction of protein levels for 26S proteasome subunits PSMA1, PMB5, and PSMC1 in wild-type mouse liver by D3T. Immunoblot analyses were performed using subunit-specific antibodies. Each sample was prepared from three pooled livers, and three similar immunoblots were obtained.

RESULTS

Levels of proteasome subunits are increased in mouse liver following treatment with D3T. Microarray analysis indicated that hepatic transcript levels of many proteasome subunits were increased following treatment of mice with D3T (Fig. 1B). Among 36 subunits of the 26S proteasome present on the Affymetrix murine genome U74Av2 GeneChip, levels of 19 subunits were increased by D3T treatment only in wild-type mice, while 5 subunits were induced by D3T treatment in both wild-type and *nrf2*-disrupted mice. Notably, transcript levels of 12 out of 14 subunits of the 20S proteasome catalytic core were increased by D3T treatment. Interestingly, gamma interferon-inducible subunits PSMB8, PSMB9, and PSMB10 were not changed by D3T treatment in either genotype. Induction of these proteasome subunits in livers obtained from vehicle or D3T-treated, wild-type and *nrf2*-disrupted mice was confirmed by RT-PCR analysis (Fig. 2A). Transcript levels of representative subunits of the 20S proteasome, namely, PSMA1, PSMA4, PSMB3, PSMB5, and PSMB6, were elevated two- to threefold 24 h after treatment with D3T in wild-type mice only. A similar pattern of increase was seen for subunits of the 19S proteasome, such as PSMC1, PSMC3, and PSMD14. Levels of proteins PSMA1, PSMB5, and PSMC1 in mouse liver were examined 24 h after treatment with D3T by immunoblot analysis. Polyclonal antibodies for each of these subunits were used for immunoblot analysis, and no nonspecific reactivity was seen. Subunits were detected at 30 (PSMA1), 22 (PSMB5), and 49 kDa (PSMC1). Hepatic levels of the PSMB5 protein were elevated 3.2-fold by D3T treatment compared to those in vehicle-treated wild-type mice, while *nrf2*-disrupted mice did not respond to treatment with D3T (Fig. 2B). Levels of proteins PSMA1 and PSMC1 in wild-type mice were increased 1.9- and 2.3-fold, respectively, following treatment with D3T, but levels in *nrf2*-disrupted mice did not increase. These results indicate that diverse subunits of the 26S proteasome are inducible, largely through a pathway that is dependent on the Nrf2 transcription factor.

Proteasome activities are elevated in D3T-treated mouse liver. Proteasome activity was measured with the fluorogenic peptides *N*-Suc-LLVY-AMC, Z-LLE-AMC, and Z-ARR-AMC as substrates to measure chemotrypsin-like, postglutamic, and trypsin-like peptidase activities, respectively. These peptidase activities could be inhibited more than 90% by the addition of 10 μM MG 132, a proteasome inhibitor (data not shown). Proteasome activity toward *N*-Suc-LLVY-AMC was elevated 2.1-fold in liver homogenates obtained from wild-type mice 24 h after treatment with D3T compared to activity in vehicle-treated controls (Fig. 3A). Smaller, but significant changes were seen with the other substrates (Fig. 3B and C). However, hepatic proteasome activity was not induced by D3T treatment in *nrf2*-disrupted mice.

PSMB5 is regulated by antioxidants and Nrf2 in murine embryonic fibroblasts. Murine embryonic fibroblasts derived from wild-type and *nrf2*-disrupted mice were treated with different phase 2 enzyme inducers to identify the effect of indirect antioxidants on the expression of the proteasome. Levels of mRNA for *PMSB5* were elevated 2.1-fold ($P < 0.05$) by treatment with either D3T or sulforaphane in wild-type fibroblasts (Fig. 4). However, the less potent inducers butylhydroxytoluene and ethoxyquin did not significantly change the level of PSMB5 mRNA. None of the inducers elevated levels of PSMB5 transcripts in *nrf2*-disrupted fibroblasts. To analyze the regulation of *PSMB5*, the promoter region (3.4 kb) was isolated from genomic DNA of mouse liver by PCR amplification and ligated into the luciferase reporter pGL3 basic vector. The murine 20S proteasome subunit β5, *PSMB5*, is located in chromosome 14 and contains several ARE-like motifs in its promoter region. Two tandem AREs were identified 341 and 52 bp upstream of the *PSMB5* gene coding region. The ARE located at the −52 position is a perfect ARE with a sequence of TGACGTCGC, while the ARE at the −341 position is TGACCAAAC, with an AC instead of GC. These candidate

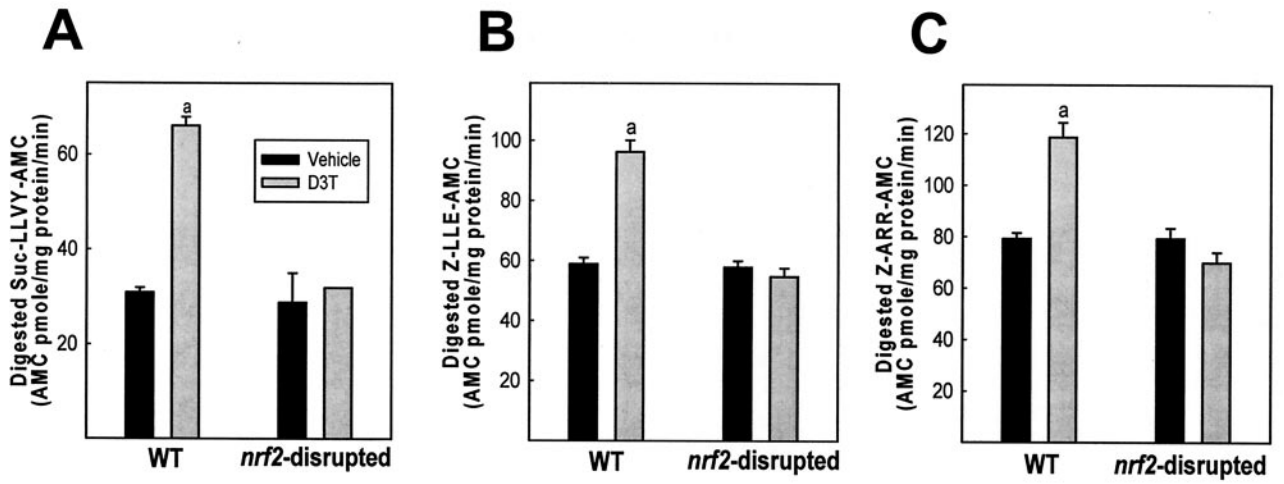


FIG. 3. Enhanced peptidase activities of proteasomes in wild-type mouse liver following treatment with D3T. Chemoptypsin-like (Suc-LLVY) (A), postglutamic (Z-LLE) (B), and trypsin-like (Z-ARR) (C) peptidase activities in liver homogenates prepared from wild-type and *nrf2*-disrupted mice were measured. Values are means ± standard errors from three experiments. a, $P < 0.05$ compared with vehicle-treated control group.

AREs are followed or preceded by inverted ARE-like sequences. Promoter truncation analysis was performed to identify the functional AREs from this promoter. The full-length promoter (−3.4kb-luc) was truncated in five different constructs, and luciferase activities from these constructs in murine embryonic fibroblasts from wild-type mice following sulforaphane treatment or Nrf2 overexpression were measured (Fig. 5A). The full-length (−3.4kb-luc) promoter was activated by sulforaphane treatment (1.5-fold), as well as by Nrf2 overexpression (2.5-fold) (Fig. 5B). Responses to sulforaphane and Nrf2 were higher (three- to fourfold) in proximal promoter constructs (−1.1kb-luc and −0.5kb-luc) than in the full-length promoter. When the proximal promoter region (−1.1 kb to −1 bp) was deleted from the full-length promoter, the resulting construct was not activated by sulforaphane treatment or Nrf2 expression [−3.4-del (−1.1)-luc]. These results suggest that a promoter containing 0.5 kb upstream of the start codon can be activated by the Nrf2-ARE pathway (Fig. 5B). However, deletion of either one of these tandem AREs [−0.2kb-luc and −0.5kb-del (−0.2)-luc] largely abolished the responses to sulforaphane and Nrf2, indicating that these two tandem AREs are necessary for the full activation of the *PSMB5* promoter by Nrf2-ARE signaling. The response of the proximal promoter of PSMB5 (−1.1kb-luc) was also measured following treatment with different antioxidants (Fig. 6A). Luciferase activity driven by the proximal promoter in wild-type cells was elevated following treatment with these antioxidants in a pattern similar to that of the induced changes in mRNA levels (Fig. 4). Sulforaphane showed the highest activation of this promoter (2.9-fold), and this activation was largely attenuated when the reporter was transfected into *nrf2*-disrupted cells (Fig. 6A). Expression of excess amounts of MafK, a repressor binding partner of Nrf2, suppressed basal promoter activity by 50% and completely blocked promoter activation by overexpression of Nrf2, as seen with other promoters regulated by AREs (32) (Fig. 6B). The enhanced constitutive promoter activity in *keap1*-disrupted cells (9.4-fold; Fig. 6B) also indicated that the promoter of *PSMB5* is regulated by Nrf2. Nrf2 accumulates in the nuclei of *keap1*-disrupted cells, leading to high basal expression of ARE-regulated genes, such as that encoding

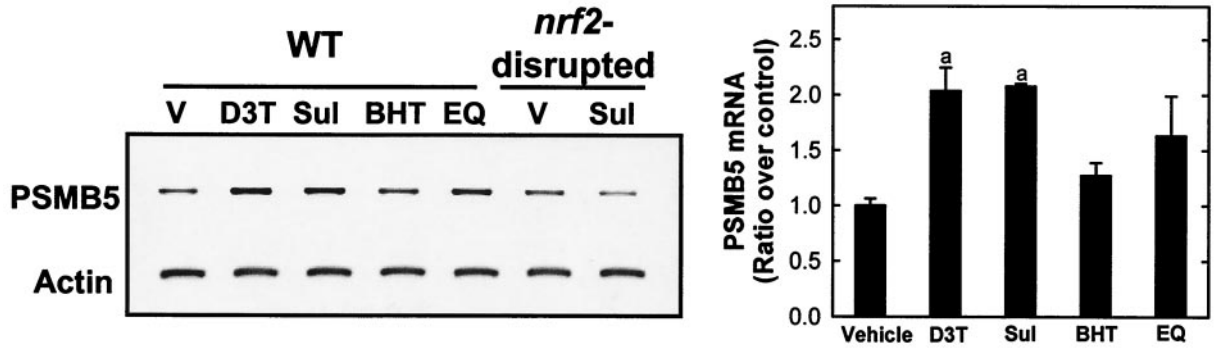


FIG. 4. Effect of antioxidants on the level of PSMB5 transcripts in murine embryonic fibroblasts. Transcript levels for *PSMB5* were measured following treatment with antioxidants D3T (30 μM), sulforaphane (Sul; 10 μM), butylhydroxytoluene (BHT; 100 μM), and ethoxyquin (EQ; 80 μM) for 18 h in fibroblasts from wild-type and *nrf2*-disrupted mice. The histogram depicts the means ± standard errors from three separate experiments. a, $P < 0.05$ compared with vehicle-treated controls.

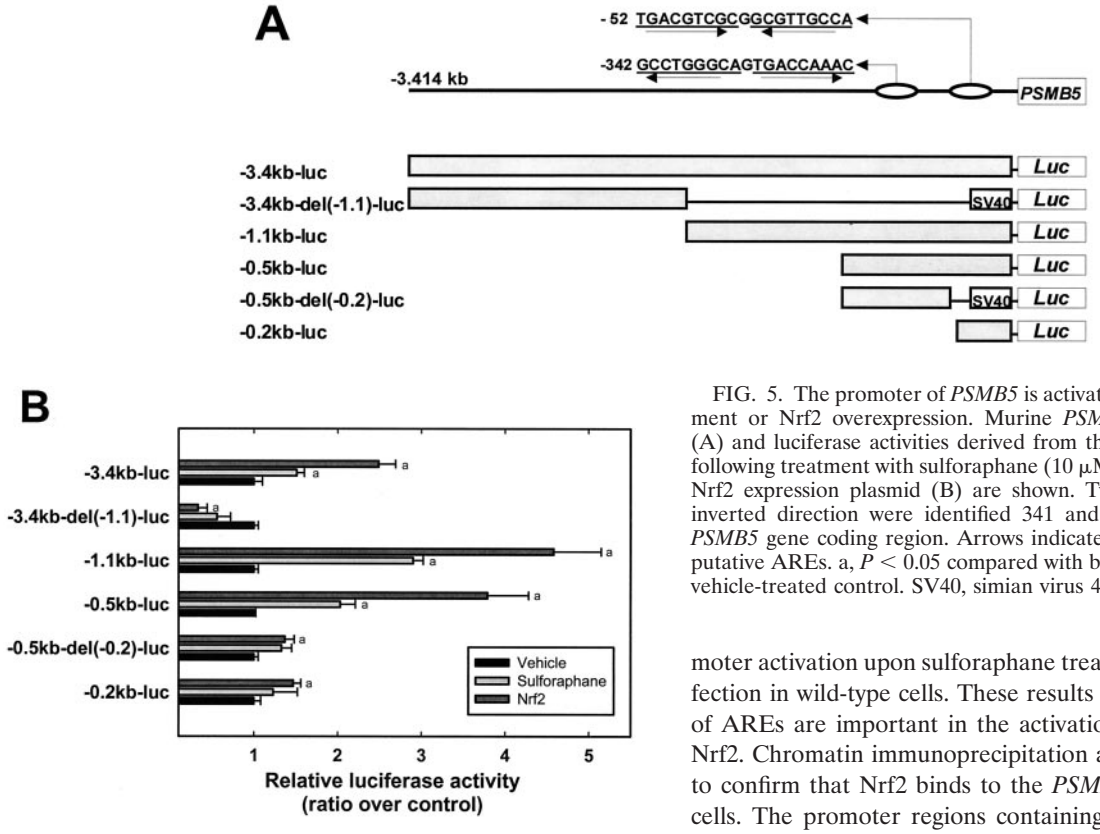


FIG. 5. The promoter of *PSMB5* is activated by sulforaphane treatment or Nrf2 overexpression. Murine *PSMB5* promoter constructs (A) and luciferase activities derived from these truncated promoters following treatment with sulforaphane (10 μM) or cotransfection of an Nrf2 expression plasmid (B) are shown. Two tandem AREs in an inverted direction were identified 341 and 52 bp upstream of the *PSMB5* gene coding region. Arrows indicate the orientation of these putative AREs. a, $P < 0.05$ compared with blank plasmid-transfected, vehicle-treated control. SV40, simian virus 40.

NQO1 (44a). Collectively, these results indicate that expression of proteasome catalytic subunit PSMB5 is elevated by the Nrf2 pathway.

PSMB5 is regulated by tandem AREs located in its proximal promoter. To confirm the results from promoter truncation, the tandem AREs were mutated. Mutations in either the −341 ARE or −52 ARE partially affected inducer responses (Fig. 7), while mutations in both tandem AREs largely abolished promoter activation upon sulforaphane treatment or Nrf2 cotransfection in wild-type cells. These results indicate that both sets of AREs are important in the activation of the promoter by Nrf2. Chromatin immunoprecipitation assays were performed to confirm that Nrf2 binds to the *PSMB5* promoter in intact cells. The promoter regions containing tandem AREs at kb −341 and −52 were detected by PCR amplification with Nrf2-immunoprecipitated chromatin from sulforaphane-treated, wild-type cells (Fig. 8A). As a positive control, the ARE of the *GSTA1* promoter, which is a well-characterized functional ARE (14), was detected in Nrf2-immunoprecipitated samples, but not in immunoprecipitants with nonspecific immunoglobulin G or GATA-1. The β-actin and *GATA-1* promoters were not amplified by the same number of PCR cycles in Nrf2-immunoprecipitated samples. Levels of binding of Nrf2 to the *PSMB5* promoter were higher in sulforaphane-treated wild-

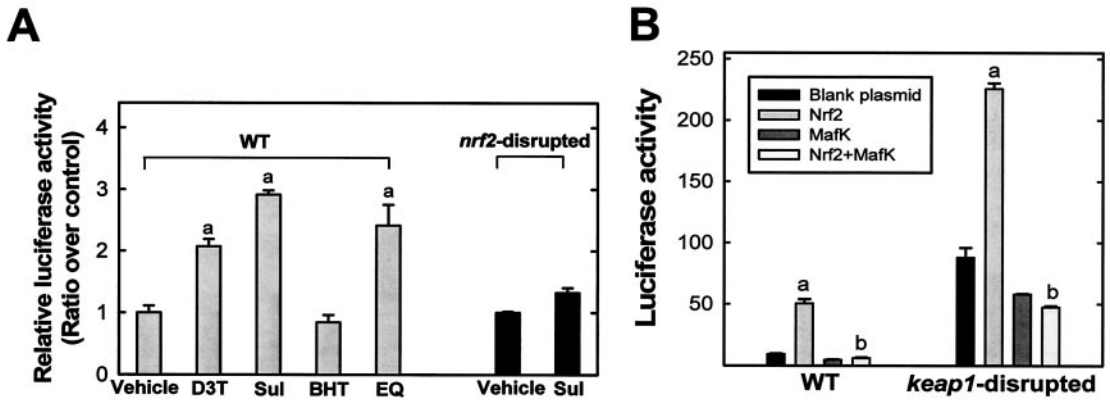


FIG. 6. The promoter of *PSMB5* is regulated by the Nrf2-ARE pathway. (A) Luciferase activity driven by the proximal *PSMB5* promoter (−1.1kb-luc) following treatment with antioxidants in wild-type and *nrf2*-disrupted cells. BHT, butylhydroxytoluene; EQ, ethoxyquin. (B) Elevated basal activity of the proximal *PSMB5* promoter (−1.1kb-luc) in *keap1*-disrupted cells. Overexpression of MafK inhibited promoter activation by Nrf2. a, $P < 0.05$ compared with blank plasmid-transfected, vehicle-treated control; b, $P < 0.05$ compared with pcDNA3-Nrf2-transfected group. WT, wild type.

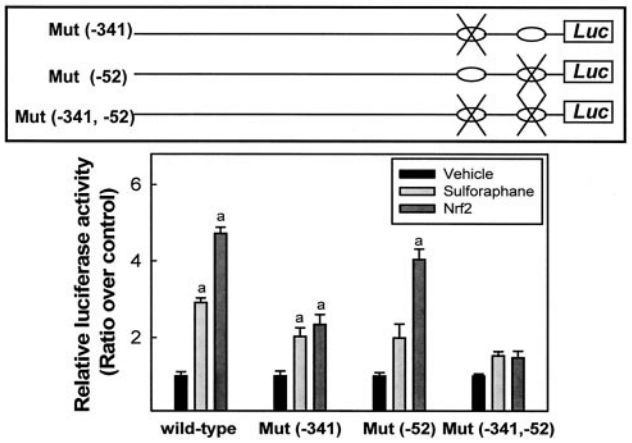


FIG. 7. Response of mutated *PSMB5* promoter to sulforaphane (Sul) treatment and Nrf2 overexpression in wild-type cells. AREs located at −341 and −52 were mutated, and singly and doubly mutated promoters were generated from the proximal promoter construct (−1.1kb-luc). Values are means ± standard errors from three experiments. a, $P < 0.05$ compared with blank plasmid-transfected, vehicle-treated control.

type cells than in vehicle-treated cells. A similar pattern of binding was observed with the *GSTA1* ARE (Fig. 8B). The *GSTA1* ARE and *PSMB5* promoters were also amplified from Nrf2-bound chromatin from *keap1*-disrupted cells, but not from *nrf2*-disrupted cells.

DISCUSSION

Oxidative stress contributes to aging and age-related diseases such as cancer, cardiovascular disease, chronic inflammation, and neurodegenerative diseases. Levels of oxidized proteins, phospholipids, and DNA increase in these processes (40). There is abundant evidence that oxidized low-density lipoprotein is involved in the formation of atherosclerotic lesions (18). Accumulation and aggregation of abnormal proteins are common features of neurodegenerative diseases; levels of oxidized and nitrated amino acids in Parkinson's and Alzheimer's diseases are high (15, 17, 34). Reduced expression of proteasome components and inhibition of proteolytic activity appear to be primary events leading to neuronal death during aging and neurodegenerative diseases (8, 17, 22). Antioxidants can prevent or retard many of the manifestations of oxidative damage. Direct antioxidants inactivate free radicals, and cell-based and animal studies demonstrate that vitamins C, E, and β-carotene are effective in preventing oxidative injury (9). Epidemiological studies have shown that consumption of diets rich in vegetables and fruits, which are excellent sources of antioxidants, are associated with reduced cancer incidence and that dietary intake of vitamin E is associated with lower risk of coronary disease (41). Paradoxically though, it has been difficult to recapitulate the protective benefits of these diets in clinical trials with defined antioxidant interventions (16). Vegetable-rich diets are also an abundant source of indirect antioxidants that, although they cannot scavenge free radicals, enhance the antioxidative capacity of cells (10). These phytochemicals increase intracellular levels of the antioxidant glutathione, boost synthesis of reducing equivalents such as NADPH, and increase the expression of enzymes, such as glutathione *S*-transferase and NQO1, that detoxify chemicals poised to generate oxidants. Indirect antioxidants can be classified into at least nine chemically distinct categories, including isothiocyanates (e.g., sulforaphane) and dithiolethiones (e.g., D3T), that nonetheless have the common chemical property of reactivity with sulfhydryl groups (10). In a few instances, exemplified by phenolic antioxidants used as food additives and polyphenols found in foods and teas, some agents can dually function as direct and indirect antioxidants (10). Many animal studies now indicate that indirect antioxidants of both natural and synthetic origin can prevent carcinogenesis, although their effects against other chronic diseases associated with aging are largely unexplored. Clinical trials have demonstrated that oltipraz, a synthetic analog of D3T, inhibits some of the early actions of the hepatocarcinogen aflatoxin B_1 while another drug, anethole dithiolethione, reduces the extent of bronchial dysplasia in smokers (28, 45). In animal models the protective actions of dithiolethiones and sulforaphane depend on the Nrf2 signaling pathway. Disruption of *nrf2* largely inhibits the induction of protective genes by these

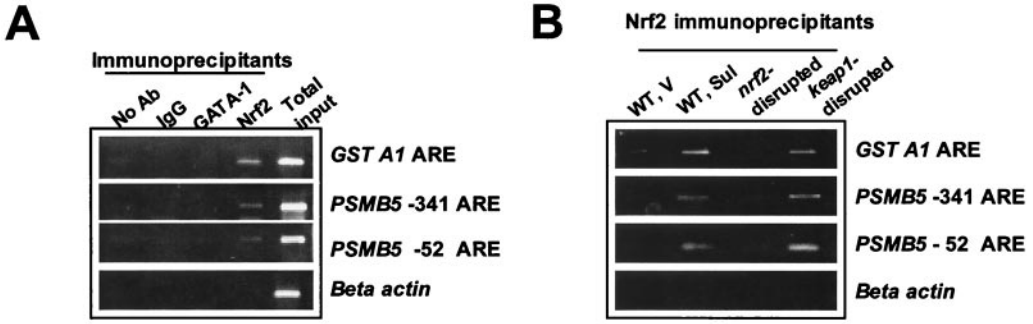


FIG. 8. Binding of Nrf2 to *PSMB5* promoter in intact cells. (A) Chromatin immunoprecipitants with no antibody (Ab), immunoglobulin G (IgG), GATA-1, or Nrf2 were used for PCR amplification of each promoter. ARE-containing promoter regions from *PSMB5* and the *GSTA1* promoter were detected in Nrf2 immunoprecipitants obtained from sulforaphane-treated wild-type cells. (B) Enhanced binding of Nrf2 to the *PSMB5* promoter following sulforaphane (Sul) treatment compared to that in vehicle (V)-treated cells. Promoter regions of *GSTA1* and *PSMB5* genes were detected in Nrf2-immunoprecipitants from *keap1*-disrupted cells, but not *nrf2*-disrupted cells. WT, wild type.

antioxidants, with resultant loss of protective efficacy against chemical carcinogenesis (25, 35, 43).

Nrf2 is a central molecular target of indirect antioxidants, and products of the genes downstream of *nrf2* are a key mammalian defense system that enables adaptation to stresses and promotes cell survival. Genes now recognized as being under the regulation of the Nrf2-ARE signaling pathway include a panel of genes encoding xenobiotic conjugating enzymes, enzymes that provide cofactors (glutathione) and reducing equivalents (NADPH) for these reactions, and antioxidative enzymes and proteins (27, 43). In this study, we demonstrate that the genes forming the 26S proteasome complex are coordinately regulated by Nrf2 in the response to indirect antioxidants. Dithiolethiones elevated transcript levels for 24 out of the 34 subunits that constitute the 26S proteasome in mouse liver; 19 out of these 24 subunits were increased in wild-type mice but not in *nrf2*-disrupted mice. Concordantly, protein levels and proteolytic activities were elevated by D3T only in wild-type mice. Promoter truncation, mutation, and chromatin immunoprecipitation studies of the murine *PSMB5* gene, which has a chemotrypsin-like proteolytic activity, further support the critical role of the Nrf2-ARE signaling pathway in the regulation of these genes.

The consensus sequence of the ARE has been proposed to be TGA(C/T)NNNGC. The first three bases from the 5′ direction are known to be critical for its activity, and the GC box is needed for maximal function (46). The ARE was identified in the promoter region of multiple phase 2 genes, and AREs from rat, mouse, and human *NQO1*, mouse and rat *GSTA1*, and human γ-glutamylcysteine ligases, are well-characterized, functional AREs. Mouse *GSTA1* has repeated AREs (underlined) in a forward direction (ATGAC ATTGCTAATGGTGACAAAGCA), and rat *NQO1* has also tandem AREs in an inverted direction (CTAGAGTCAC AGTGACTTGGCA) (12, 14, 33). One of the AREs from NQO1 contains the complete consensus sequence of the ARE, while the other has no GC box. Both elements of the tandem AREs of *GSTA1* and *NQO1* are essential for the response of these promoters to enzyme inducers and Nrf2 expression (14, 33). By contrast, human γ-glutamylcysteine ligases have a single functional ARE (TGACAAGC in the regulatory subunit and TGAC TCAGC in the catalytic subunit) in their promoters (33). The murine *PSMB5* gene has tandem AREs in an inverted direction that are similar to the rat *NQO1* ARE. From the results of promoter truncation and mutation analyses, the two tandem AREs in the *PSMB5* promoter appear to be important for the maximal response of this promoter to Nrf2-mediated signaling. Many other subunits of the murine proteasome have putative AREs in their 5′-flanking region. In addition, the promoters of rat and human *PSMB5* have several ARE motifs. Levels of protein PSMB5 in rat tissues can also be elevated following treatment with D3T (data not shown).

The ubiquitin-proteasome pathway maintains cellular homeostasis by regulating proteins involved in signaling and cell cycle pathways. Nrf2 is a substrate for the ubiquitin-proteasome system. Treatment of cells with a proteasome inhibitor prevents rapid degradation of this protein, leading to enhanced expression of the downstream gene encoding γ-glutamylcysteine ligase (37). Recently, Itoh et al. (21) proposed that Nrf2 protein turnover is regulated by Keap1-mediated subcellular compartmentalization of this transcription factor. In our study, D3T induced the expression of a broad range of proteasome subunits encompassing both the catalytic core (20S proteasome) and the ATP-dependent regulatory core (19S proteasome). Twelve out of 14 of the subunits of the 20S proteasome were induced. By contrast, D3T increased the expression of just a few of the ubiquitination enzymes in mouse liver (27). Many studies have demonstrated that the 20S proteasome can directly degrade oxidatively damaged proteins without assistance from the ubiquitination process by direct recognition of a hydrophobic patch derived from oxidation (6). Mutational inactivation of the E1 ubiquitin-activating enzyme does not affect the degradation of oxidized proteins by proteasomes. Therefore, the enhanced expression of multiple subunits of the 26S proteasome, and in particular its 20S catalytic core, by antioxidants might facilitate the removal of damaged proteins, without disturbing physiologic regulation of other proteins. This action may function to attenuate or perhaps prevent progression of human diseases related to oxidative stress damage. Expression of β-subunits of the proteasome is repressed during aging, and this is reflected in increased levels of oxidized and ubiquitinated proteins within cells. A recent report has shown that proteasome expression in senescent cells is downregulated and that stable expression of PSMB5 by transfection reversed the phenotype of senescence and led to enhanced resistance to oxidative stresses (5).

Regulation of the mammalian proteasome is not well understood. Direct oxidative modification of the catalytic core subunits of the proteasome inhibits their activities (7). There are few reports concerning the regulation of expression of proteasome subunits in mammalian cells. Immunoproteasomes are inducible by chemical treatment in animal cells. Expression of PSMB8, PSMB9, and PSMB10 is enhanced by gamma interferon and lipopolysaccharide exposure (13, 48). Takabe et al. (42) reported that the antiatherogenic antioxidant probucol repressed expression of PSMA2, PSMA3, and PSMA4. Lee et al. (29) showed that overexpression of the antiapoptotic protein BCL-2 increased proteasome activity in animal cells. Very recently, Meiners et al. (31) demonstrated that the proteasome inhibitor MG132 increases the expression of a broad range of subunits of the proteasome in mammalian cells. In *Saccharomyces cerevisiae*, expression of the 26S proteasome subunits is coordinately regulated by the transcription factor Rpn4p (30, 47). Rpn4p is a C_2H_2-type finger motif protein that regulates basal expression of yeast proteasome subunits by transactivating proteasome-associated control elements (5′-GGTGGCAA A-3′) in their promoters. Expression of this protein can be upregulated by Pdr1p and Yap1p under conditions of stress. While Nrf2 appears to be a universal transcription factor for the upregulation of proteasome subunits by antioxidants in mammalian cells, Nrf2 and Rpn4p have no apparent homology.

Collectively, our results indicate that the 26S proteasome is one of several target gene categories regulated by the transcription factor Nrf2 that can contribute to protection against oxidative stress. Induction of these protective pathways provides efficient means for cells to survive conditions of stress that result from endogenous processes (e.g., inflammation) or exogenous ones (e.g., environmental pollutants) that collec-

tively enhance the burden of chronic disease. Induction of these pathways by indirect antioxidants through dietary or pharmacological means provides opportunities for broad-ranging protection in settings where supplementations with direct antioxidants have had limited benefit.

ACKNOWLEDGMENTS

This work was supported by grants CA39416 and CA94076 from the National Institutes of Health and Center grant ES03819.

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